Interaction of Zwitterionic Penicillins with the OmpF Channel Facilitates Their Translocation

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ABSTRACT To study translocation of β-lactam antibiotics of different size and charge across the outer bacterial membrane, we combine an analysis of ion currents through single trimeric outer membrane protein F (OmpF) porins in planar lipid bilayers with molecular dynamics simulations. Because the size of penicillin molecules is close to the size of the narrowest part of the OmpF pore, penicillins occlude the pore during their translocation. Favorably interacting penicillins cause time-resolvable transient blockages of the small-ion current through the channel and thereby provide information about their dynamics within the pore. Analyzing these random fluctuations, we find that ampicillin and amoxicillin have a relatively high affinity for OmpF. In contrast, no or only a weak interaction is detected for carbenicillin, azlocillin, and piperacillin. Molecular dynamics simulations suggest a possible pathway of these drugs through the OmpF channel and rationalize our experimental findings. For zwitterionic ampicillin and amoxicillin, we identify a region of binding sites near the narrowest part of the channel pore. Interactions with these sites partially compensate for the entropic cost of drug confinement by the channel. Whereas azlocillin and piperacillin are clearly too big to pass through the channel constriction, dianionic carbenicillin does not find an efficient binding region in the constriction zone. Carbenicillin's favorable interactions are limited to the extracellular vestibule. These observations confirm our earlier suggestion that a set of high-affinity sites at the narrowest part of the OmpF channel improves a drug's ability to cross the membrane via the pore.

INTRODUCTION

The growing number of pathogenic bacteria that display resistance to multiple antibiotics is becoming an increasingly worrying clinical problem (1–4). These days, a ''good'' antibiotic is expected to exhibit a high affinity for its target and to reach it rapidly, escaping modification by inactivating enzymes or expulsion by active efflux mechanisms (5).

Compounds of the β-lactam family belong to the most important and most widely used group of antibacterial agents. They interfere with the biosynthesis of the peptidoglycan, a complex polymer of sugars and amino acids constituting the major component of the bacterial cell wall. The antibacterial efficacy of the β-lactam family of antibiotics is dependent on their structural similarity to the substrate of the DD-transpeptidases located in the periplasmic space (6). Inhibition of these enzymes by β-lactams leads to structural instability and death of the bacteria.

Before reaching the target, β-lactam antibiotics have to cross the outer membrane. The general diffusion porin OmpF (outer membrane protein F) is considered to be the primary gateway for these antibiotics (7,8). Indeed, some β-lactam-resistant strains of *Escherichia coli* have shown a deficiency in OmpF expression (9). Mutations leading to structural alterations of OmpF porins, such as a decrease in the pore radius, seriously inhibit antibiotic uptake (10,11). Therefore, better knowledge of the molecular basis of drug penetration via the general porin OmpF is an important element of the comprehensive picture of bacterial resistance.

The rate of β-lactam antibiotic penetration depends on many factors, including the charge distribution on the antibiotic molecule (12–14). Particularly, studies with reconstituted proteoliposomes (13–14) and intact cells (15) revealed that zwitterionic compounds penetrate very rapidly as compared with monoanionic β-lactams. At the same time, the introduction of the second negative charge on the solute resulted in further reduction of the translocation rates.

Recently we incorporated individual OmpF channels into planar lipid membranes and investigated the transport of a β-lactam antibiotic, ampicillin (16). In analogy to substrate-specific channels such as the sugar-specific maltoporin (17–19), the interaction between the permeating ampicillin and the channel pore turned out to be strong enough to be seen as time-resolved blockages of the small-ion currents through the channels. It was shown that the number of blockage events per second is a strong function of the bathing solution pH with a sharp peak at about pH 4.5 (16). Regulation of the binding rate by the solution pH can be qualitatively understood by taking into consideration the ampicillin acid-base

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equilibrium. According to ampicillin's pK values, the fraction of zwitterionic form reaches its maximum at approximately pH 5, whereas cationic or anionic species predominate in solution at lower or higher pH values (20). These results suggest that the zwitterionic form of ampicillin interacts with the charged or polar residues inside the channel and that this interaction is favorable for the high translocation rates found by Nikaido and colleagues (12–14). This finding can also explain the in vivo experiments of Rolinson and Stevens (21) indicating that ampicillin is ~10 times more active toward strains of *E. coli* in pH 5.5 than in pH 8 buffer.

The availability of the high-resolution structure of OmpF (22) and the knowledge of the charge distribution within the molecule (23–26) allow detailed molecular modeling of the channel. Molecular dynamics (MD) simulations have been particularly powerful in gaining insights into OmpF properties (27–29). Computer simulations were used to study transport of ions through the channel to unveil the physical basis of its selectivity and conductance (24,30–34).

Despite the fact that standard MD simulations are widely used for studying ligand-protein interactions, this simulation method only samples a restricted region around local minima in the free energy surface. Procedures have been developed to efficiently explore rough energy profiles of complex systems (35,36). Nonequilibrium steered MD simulations were successfully employed in the diffusion of molecules through protein channels, such as glycerol conduction by aquaglyceroporin GlpF (37) and translocation of alanine and methylglucose through OmpF pores (38). In a previous study (39) that used an MD algorithm capable of investigating rare events (36), the permeation of ampicillin was explored. In this procedure, a history-dependent potential term was injected into the system to accelerate both the diffusion of the antibiotic molecules along the channel axis and the formation and breakage of hydrogen bonds between the drug and the protein. A microscopic mechanism for this process was proposed. An electrostatic environment favorable for ampicillin penetration was found in the pore, whereas the translocation process was shown to be dominated by steric hindrance. Once in contact with the pore, the flexibility of the ampicillin molecule allows it to change its orientation and to pass through the pore. Molecular modeling (16) also suggests that the charge distribution of the ampicillin molecule complements the charge distribution of the narrowest part of the OmpF porin. Interaction between these charges creates a region of attraction that facilitates drug translocation through the constriction zone and results in higher permeability rates for ampicillin at pH 5.5 compared with pH 8 (21).

Here we extend our study to examine new aspects of the structure-function relationship for the OmpF-facilitated translocation of different β-lactams of varying antibacterial potency. We deal only with the initial step in the chain of events involved in the drug action—namely, drug penetration into Gram-negative bacteria. Following Yoshimura and Nikaido (14), we investigate several penicillins selected on the basis of their size (ampicillin, amoxicillin, and carbenicillin versus larger piperacillin and azlocillin) and charge (zwitterionic compounds versus monoanionic and dianionic compounds) rather than on the basis of their chemotherapeutic utility. The interaction between the permeating drug and the OmpF pore is probed by analyzing drug-induced current fluctuations. To reach a clear understanding of the molecular forces involved in drug translocation, we use history-dependent MD simulations at an all-atom level. A wide region of space inside the pore could be explored in a time compatible with MD simulations (a few nanoseconds). Since the drug passage is dominated by steric hindrance, we focus our MD analysis on the smaller antibiotics amoxicillin, ampicillin, and carbenicillin.

MATERIALS AND METHODS

Chemicals

The following chemical reagents were used in this study: NaCl, NaOH, and HCl (Mallinckrodt, St. Louis, MO); ultrol grade MES or HEPES (Calbiochem, San Diego, CA); ''purum'' hexadecane (Fluka, Buchs, Switzerland); 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL); pentane (Burdick and Jackson, Muskegon, MI); agarose (Bethesda Research Laboratory, Gaithersburg, MD). Doubly distilled and deionized water was used to prepare solutions. After preparation, all solutions were purified by filtration through a 0.45-μm filter.

β-lactam antibiotics were purchased from Sigma as a sodium salt (azlocillin, piperacillin), disodium salt (carbenicillin), or monohydrates (ampicillin, amoxicillin). The antibiotic stock solutions were made at the concentration of 5.7 mM in 1 M NaCl buffered by 5 mM MES or HEPES. Control experiments at pH 5–7 showed no dependence of the OmpF properties (conductance and open channel noise) on the presence of the buffers. In contrast to our previous study (16), we used NaCl instead of KCl as a bathing electrolyte because many β-lactams are manufactured as sodium salts. Following Yoshimura and Nikaido (14), we used NaOH to facilitate dissolving zwitterionic β-lactams.

Reconstitution experiments and noise analysis

Reconstitution experiments and noise analysis have been performed as described in detail previously (40). To form planar lipid bilayers with the lipid monolayer opposition technique (41), we used a 5% solution of diphytanoylphosphatidylcholine in pentane. A Teflon cell with a 70-μm-diameter aperture in the 15-μm-thick Teflon partition and silver-silver chloride electrodes with agarose bridges were described previously (42). The total capacitance of the cell was 50–60 pF, and the film capacitance was close to 25 pF. Small amounts of wild-type OmpF from a diluted stock solution of 1 mg/ml containing 1% (v/v) of Octyl-POE from Alexis, Switzerland, were added to the *cis* side of the chamber. Spontaneous channel insertion was usually obtained within a few minutes while stirring at the applied voltage from −180 to −200 mV. Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage clamp mode. Signals were filtered by a low-pass 8-pole Butterworth filter (Frequency Devices, Haverhill, MA) at 15 kHz and recorded simultaneously by a video cassette recorder operated in digital mode and directly saved into the computer memory with a sampling frequency of 50 kHz. Amplitude, probability, and noise analyses were performed using software developed in-house.

Simulation methodology

Our model is based on the three-dimensional structure of the wild-type OmpF resolved at 2.4 Å (22). Both theoretical and experimental investigations

conducted on OmpF have shown mutual independence of the three subunits (no cooperativity between the three monomers) for ion (32) and small-molecule transport (38,43). The absence of cooperativity between the three monomers and the rigidity of the structure exclude low-frequency breathing modes as an important contribution to the channel dynamics and make MD simulations a feasible tool to investigate antibiotic translocation at the microscopic level. We focus on a single monomer of OmpF that we immerse in a micelle-like environment formed by 100 molecules of lauryl dimethyl amino oxide (LDAO) detergent and hydrated by 7600 water molecules. The detergent, generally used to crystallize membrane proteins, provides a natural hydrophobic environment that prevents direct interactions between the external protein surface and water. The system was prepared as described earlier (39), all residues being in their standard ionization state corresponding to pH 7 except for Glu-296 and Asp-312, which were protonated; counterions were introduced to neutralize protein charges. We used the AMBER force field for OmpF, the detergent (44), and the three antibiotics and TIP3P for water. All simulations were performed, after relaxation, at constant volume and temperature using state-of-the-art software (45).

Details of the metadynamics algorithm to investigate rare events have been described previously (36,39). Briefly, to overcome the timescale problem we introduce artificially a time-dependent parameter to modify the underlying free energy during the simulation. This creates a component to the forces discouraging the molecule from revisiting the same place and drives the molecule through the channel. This parameter acts only on a few slowly changing variables that describe the translocation process, making them faster on the MD simulation timescale. Here we selected two such slow variables, the position of the drug along the z axis and the number of hydrogen bonds between the drug and the channel.

RESULTS

We concentrated on the molecular process of β-lactam antibiotic penetration into the periplasmic space of *E. coli*. Previously it was shown that ampicillin interacts with the bacterial channel OmpF in a way that renders the drug's translocation events time-resolvable (16). The goal of the current study is to understand whether such interaction is a general feature of other penicillins active against *E. coli*. To achieve this goal, we probed various commercial penicillin antibiotics differing in size, charge distribution, and hydrophobicity.

Table 1 shows the schematic structures and physicochemical properties of the antibiotics used in this work. The antibiotics similar to ampicillin in size are amoxicillin and carbenicillin, whereas azlocillin and piperacillin have bulky lateral chains (compare columns 2 and 3). Ampicillin has two ionizable groups, an acid function on the penam ring and a primary amine on the lateral chain (columns 3 and 4). The antibiotic presenting the same charge distribution as ampicillin is amoxicillin. In fact, the biological activity of β-lactam antibiotics and their hydrophobicities have been shown to be interrelated (13,14,46). Here we list the standard penicillin hydrophobicity values (column 5) normalized to a common octanol-water partition log P (logarithm of partition coefficient) scale as given by Petrauskas and Svedas (46). Compared to ampicillin, the hydrophobicity of the lateral chain for amoxicillin is reduced by the OH group. The last column in Table 1 shows the relative diffusion rate of the drugs reported by Yoshimura and Nikaido (14).

Experiments with reconstituted OmpF

First, we quantified the interactions of the five different β-lactams with the OmpF channel in reconstitution experiments. We analyzed antibiotic-induced fluctuations in the small-ion current through single porins at the symmetrical drug addition. Fig. 1 *A* shows that at pH 5 ampicillin and amoxicillin produced time-resolved current interruptions reflecting the reversible occlusions of single subunits of the trimeric OmpF channel. In contrast, over the pH interval from 4 to 7, carbenicillin, azlocillin, and piperacillin produced only a negligible effect on the ion flow through the channel (Fig. 1 *B*). As in the previous study (16), the number of blockage events for ampicillin and amoxicillin was found to be a strong function of pH (data not shown). The maximum rate is achieved at approximately the same pH values as the pH corresponding to the maximum in the zwitterionic form of the drug molecules in water solutions (20).

Statistical analysis of the characteristic times in the unblocked and blocked states gives the on and off rate for the drug binding. For convenience, the time-dependent fluctuating current can be transformed into a frequency domain using power spectrum analysis (47). In Fig. 2, we show typical spectra of the drug-induced current fluctuations. Addition of ampicillin and amoxicillin generate an excess noise whose power spectra can be described by single Lorentzians (Fig. 2 *A*). At -100 mV of applied voltage the low-frequency parts of the spectra exceed those of a channel in pure salt solution by $\sim$2 orders of magnitude. Earlier studies showed that this noise could be used to obtain the characteristic transport parameters (42,48,49).

Fitting a spectrum to the Lorentzian, $S(f) = S(0)/(1+(f/f_c)^2)$, where $S(0)$ is the low-frequency spectral density and f_c is the so-called corner frequency, gives the relaxation time constant as

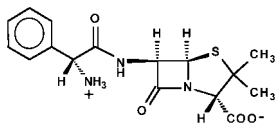
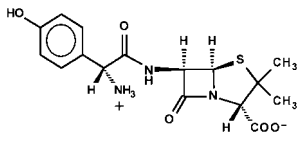
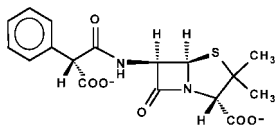
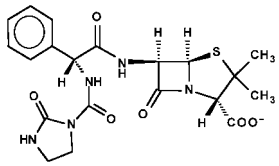
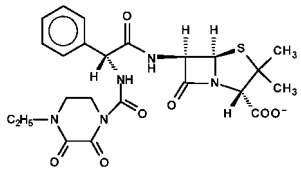
$$\tau = (2\pi f_c)^{-1} = (k_{on}[c] + k_{off})^{-1}, \quad (1)$$

where k_{on} and k_{off} are the on and off rate constants of the drug molecule binding and $[c]$ is the drug concentration in the membrane-bathing solution. At $[c] \ll k_{off}/k_{on}$ (which is the case here), the characteristic time is close to the average time of drug residence in the channel $\tau \approx \tau_r = k_{off}^{-1}$. The fitted values of τ at -100 mV applied voltage are 120 μs for ampicillin and 87 μs for amoxicillin.

Fig. 2 *B* shows that carbenicillin, azlocillin, and piperacillin induce no or only a small increase in the noise spectrum. This suggests (50,51) that the OmpF porin is not permeable or is poorly permeable for these molecules.

Fig. 3 *A* demonstrates the voltage dependence of the low-frequency spectral density of the excess noise for amoxicillin and ampicillin. If current noise is produced by the voltage-independent conductance fluctuations, the power spectral density is supposed to be proportional to the square of the voltage (47,52). This is obviously not the case here: Fig. 3 *A*

TABLE 1 Structure and physicochemical properties of the compounds used

β-lactam generic name	Molecular mass (Da)	Structural formula	Electrical charge (at pH 5)	Standard hydrophobicity (46)	Relative diffusion rate through OmpF (14)
Ampicillin	349		+/–	−0.098	43–49
Amoxicillin	365		+/–	−0.743	—
Carbenicillin	376		–/–	−0.820	2–8
Azlocillin	460		–	—	—
Piperacillin	517		–	0.450	<5

shows that the spectral density recalculated to 100 mV as $S(0) \times (V/100\ \text{mV})^{-2}$, where V is the applied voltage, is not constant (though indeed finite in the limit of small voltages). The recalculated value of $S(0)$ exhibits a maximum at positive voltages for both ampicillin and amoxicillin, indicating channel structural asymmetry. Therefore, conductance fluctuations and the underlying processes of drug exchange between the bulk and the channel do depend on the applied voltage. The mechanism of this voltage sensitivity is not clear at the moment. It could be related to the dipolar moment of the antibiotic molecules, but not necessarily so, since the binding of neutral sugar molecules to maltoporin channels was found to be voltage-dependent as well (17,18). A possible explanation is that the channel-forming protein molecule gets elastically distorted by the applied electric field acting on its charges and dipoles.

It can be shown (16) that the number of blockage events per second, the on rate of drug binding to the OmpF trimer, can be related to $S(0)$ as

$$\nu = \frac{S(0)}{4(\Delta g)^2 \tau^2 V^2}, \tag{2}$$

where Δg is the conductance change induced by one antibiotic molecule (here, 1/3 of the total trimeric channel conductance). Inspection of ν and τ_r as functions of V allows us to investigate their individual contribution to $S(0)$ at different voltages. Over the whole range of applied voltages, we find that the ampicillin residence time has a maximum of $\sim$300 μs at $V \sim$100 mV, whereas this behavior is not observed for amoxicillin (Fig. 3 *B*). Interestingly, the number of blockage events per second induced by ampicillin and amoxicillin binding is approximately a linear function of the

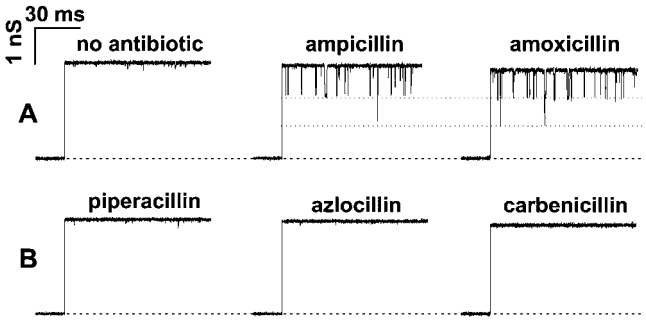


FIGURE 1 Typical tracks of ion currents through single trimeric OmpF channels reconstituted into planar lipid membranes in the presence of zwitterionic and anionic penicillins. Membrane bathing solutions contained 1 M NaCl (pH 5.0) and 5.7 mM of the indicated antibiotic, and the applied voltage was −100 mV. Time resolution was 15 μs. (*A*) Penetrating zwitterionic (ampicillin or amoxicillin) amino penicillins modulate ion current through OmpF. In the absence of antibiotic (*left*), the ion current is mainly determined by the geometry and surface properties of the channel pore. The current is stable; no high-amplitude interruptions are seen. In the presence of ampicillin (*middle*) or amoxicillin (*right*), one of the three OmpF pores gets spontaneously blocked by a translocating drug molecule. At high time resolution these blockages are seen as well-defined steps to 2/3 of the open channel current and back. (*B*) Anionic penicillins do not significantly affect ion current through OmpF. No interruptions in the current can be seen in the presence of piperacillin (*left*), azlocillin (*middle*), or carbenicillin (*right*).

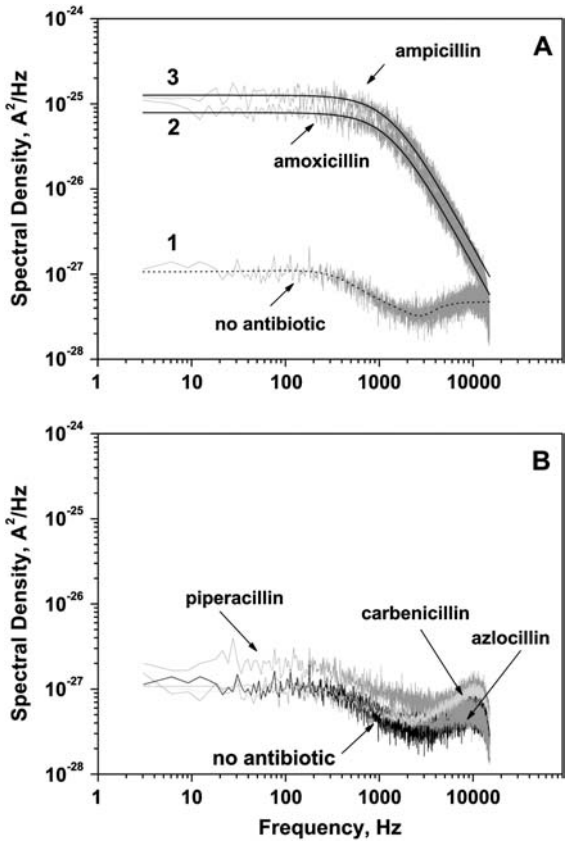


FIGURE 2 (*A*) Power spectral densities of the noise in the current through a single fully open OmpF channel in the presence of zwitterionic penicillins at −100 mV applied voltage exceed those obtained in pure 1 M NaCl solution (pH 5.0) by ~2 orders of magnitude. Smooth solid lines through the spectra are Lorentzians with $\tau = 1.2 \times 10^{-4}$ s for ampicillin- and 8.7×10^{-5} s for amoxicillin-containing solutions. (*B*) Spectral densities of OmpF current noise in the presence of anionic penicillins (see Fig. 1 *B*) are very close to those obtained in pure 1 M NaCl solutions. The effect of these drugs is small and is difficult to separate from the noise related to reversible ionization of the channel residues (40). Carbenicillin and azlocillin do not produce any measurable noise at all. The noise spectrum in the presence of piperacillin can be approximated by a sum of the spectrum in the penicillin-free solution and a frequency-independent, white component extending to frequencies above 10 kHz. This suggests that piperacillin's residence time on a site in the channel is in the range of microseconds or shorter. Therefore, fluctuation analysis shows that the anionic antibiotics in Table 1 bind to the narrowest part of the OmpF channel pore either not at all (azlocillin and carbenicillin) or only weakly (piperacillin).

applied voltage (Fig. 3 *C*). This rate is ~4 times higher at −200 mV than at + 200 mV for both antibiotics. Recalling that in these experiments we use symmetrical drug addition (antibiotics are added to both sides of the membrane in equal concentrations), this finding illustrates OmpF channel asymmetry.

Molecular dynamics simulations of penicillin binding

According to the high-resolution three-dimensional structure of OmpF (22), the porin forms three water-filled channels per trimer, the functional unit of the protein in the outer membrane. In each monomer, 16 β-strands span the outer membrane and form a barrel with short turns at the periplasmic side and large loops at the outside of the cell. Unlike the other loops, the third loop, L3, folds into the barrel, forming a constriction zone at half the height of the channel. Therefore, this loop contributes significantly to channel permeability properties, such as its exclusion limit and selectivity. At the constriction zone, there is a strong transverse electrostatic field that is produced by acidic residues in loop L3 and a cluster of basic residues at the barrel wall opposite the loop.

First, we investigated the structural perturbations of the protein during diffusion of ampicillin along the z axis. We started our simulation for influx (from the extracellular to the intracellular side of the channel) by placing the ampicillin molecule above the constriction, at $z = 7$ Å (the position of the center of mass; for comparison, Arg-82 is positioned at $z = 2$ Å). In the efflux (from the intracellular to the extracellular side of the channel) simulation, the starting position was $z = -3$ Å. Along these two pathways we calculated the instantaneous value of the root mean-square displacements of the whole protein backbone (Fig. 4, *black trace*) and of the amino acids of loop L3 (Fig. 4, *red trace*) from the published x-ray structure. As can be seen, during efflux (*upper panel*) and influx (*middle panel*), the displacements of the protein backbone are <1.8 Å on average, demonstrating that during the translocation the protein structure

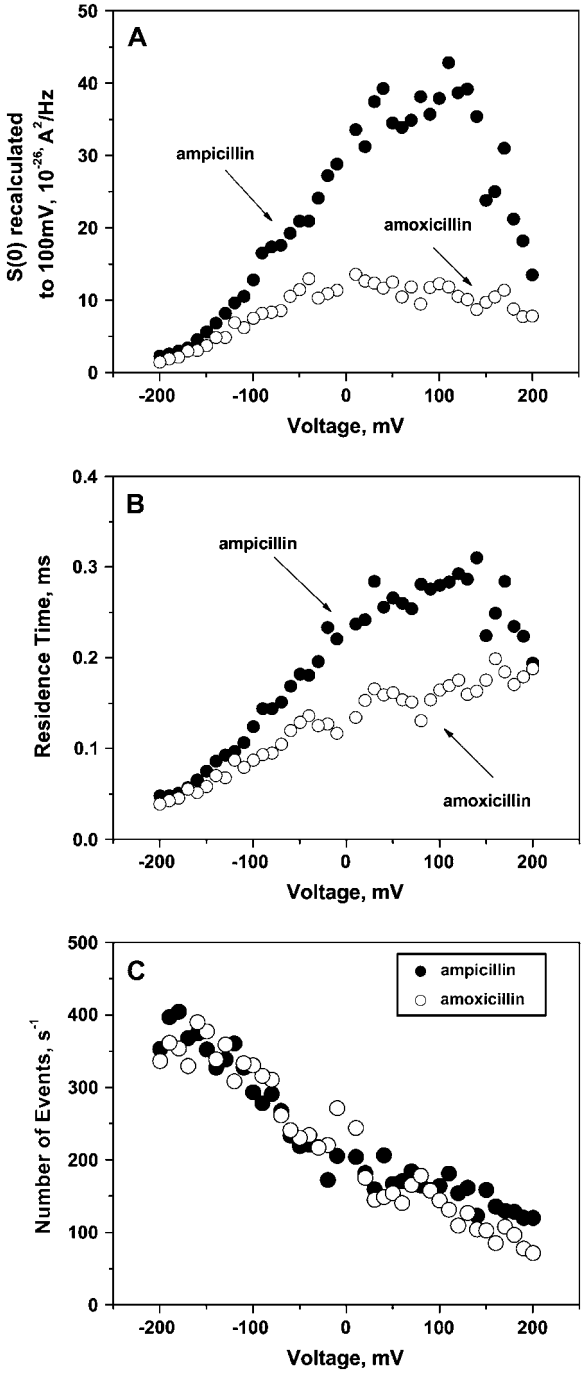


FIGURE 3 (*A*) Low-frequency power spectral density of the ampicillin- (●) and amoxicillin (○)-induced current fluctuations normalized to 100 mV. (*B* and *C*) The residence time (*B*) and number of events (*C*) calculated from fitting fluctuation spectra to Lorentzians (Fig. 2 *A*). All three of these parameters depend on the applied voltage. Note that even though the number of blockages is practically the same for both drugs at any given voltage in the studied range, the residence time differs significantly.

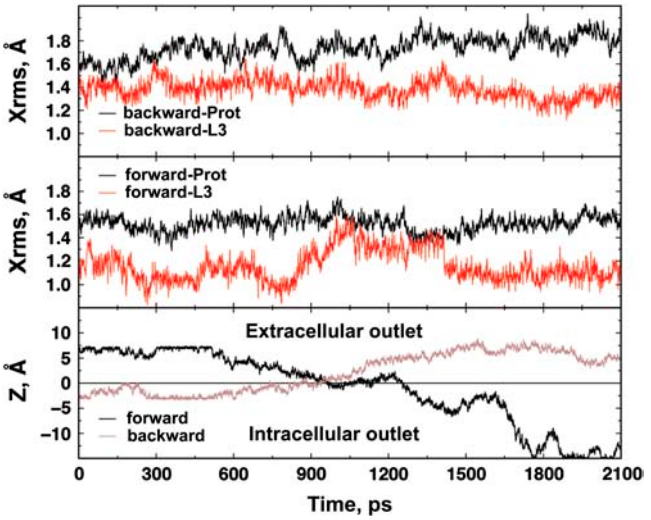


FIGURE 4 Instantaneous root mean-square deviations of the whole protein backbone (*black*) and L3 loop (*red*) compared to the x-ray structure during efflux (*upper panel*) and influx (*middle panel*) processes. Instantaneous center-of-mass positions of ampicillin along the *z* axis of the channel during the metadynamics simulations probing influx and efflux processes (*bottom panel*).

is maintained. It is interesting to note that the displacements of loop L3 are even less than the displacements of the protein backbone. During influx, a small jump of ~0.2 Å was visible whereas during efflux the average distance was almost constant, ~1.4 Å. In the bottom panel of Fig. 4, we show the corresponding position of the center of mass of ampicillin along the *z* axis during the influx and efflux translocation processes. These findings suggest that pore radius fluctuations are too small to accommodate the permeation of larger molecules. Moreover, the stericity plays a key role in the translocation (39). It is tempting to postulate, therefore, that the low permeability of OmpF toward azlocillin and piperacillin, the monoanionic compounds of larger size (Table 1), probably originates from their bulky lateral chains.

Ampicillin visits many conformations in the upper (extracellular) region of the channel covering a range of 3–4 Å along the pore axis (39). Near the constriction zone the molecule is mainly oriented parallel to the transversal electric field of the pore. Contact points for this conformer with the central OmpF residues are highlighted in Fig. 5: NH_3^+ interacts with Glu-117, COO^- with Arg-82 and Arg-132, and the phenyl group finds a favorable environment near Tyr-22 and Tyr-40, or Tyr-22, Tyr-32, and Phe-118. Zwitterionic amoxicillin and dianionic carbenicillin have dimensions similar to ampicillin (Table 1), so that the difference in characteristic behavior observed in Fig. 1 may come from specific interactions with the residues near the constriction zone.

Both standard and metadynamics MD simulations were performed for amoxicillin and carbenicillin to explore the possibility of these drugs to occupy the region near the constriction zone and to block the passage of ions. As a starting point we used the conformation of ampicillin above the constriction zone at $z = 7$ Å, as in Fig. 5. Then we transformed ampicillin to amoxicillin and carbenicillin, adding an OH group to the phenyl ring and replacing NH_3^+ with COO^-,

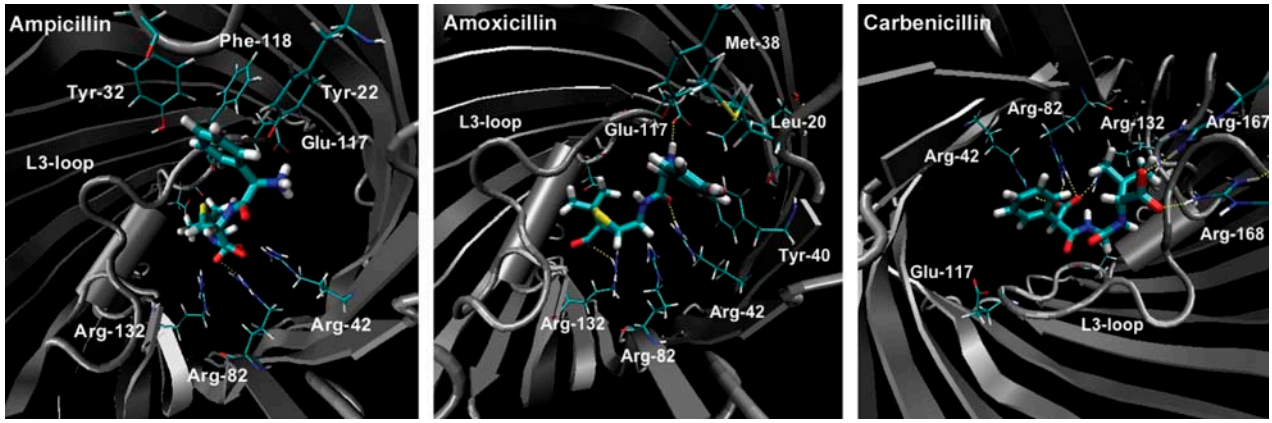


FIGURE 5 Top view of the most probable conformers for ampicillin (*left*), amoxicillin (*middle*), and carbenicillin (*right*) extracted from MD simulations. For clarity, the cartoons show only those protein side chains whose interactions with the antibiotics were identified as significant. The figures were produced with VMD 1.8.1 (61). Carbon atoms are colored by cyan, hydrogen atoms by white, nitrogen atoms by blue, oxygen atoms by red, and sulfur atoms by yellow.

respectively. Two standard MD simulations of 6 ns each were used to relax these conformations. Although standard MD simulations do not allow determination of the most stable positions in such a short time, we observed a very different behavior of carbenicillin with respect to ampicillin and amoxicillin. The addition of the hydroxyl group to transform ampicillin into amoxicillin does not completely change the interactions in the upper region. Nevertheless, amoxicillin shows a higher stability, with fluctuations of the order of only 2–3 Å. This difference is due to the reduced hydrophobicity of amoxicillin (Table 1). Whereas the carboxylate and ammonium groups can simultaneously interact with the charged residues Arg-42, Arg-82, and Arg-132 and Glu-117 of OmpF, respectively (Fig. 5), the aromatic group finds favorable hydrophobic environments with Tyr-22, Tyr-40, Met-38, and Leu-20. The additional OH group forms H-bonds with water molecules inside the channel and with amino acids Met-38 (CO group and sulfur atom) and Gln-66 (NH_2 group). These additional interactions improve the stability in this region with respect to ampicillin. It is important to note that in both ampicillin and amoxicillin conformations the free space available for permeating electrolyte ions is too small. This finding is in agreement with the nearly complete monomer closures induced by ampicillin binding (Fig. 1 *A*).

The substitution of NH_3^+ with COO^- in carbenicillin changes the binding pattern more significantly. In contrast to the drugs discussed above, once placed at $z = 7$ Å, carbenicillin moves to the upper part of the pore and reorients parallel to the channel axis with the two carboxylate ends interacting with Arg-82 and Arg-132 (COO^- on the lateral chain) and the external Arg-167 and Arg-168 (COO^- on the penam ring) (Fig. 5). Interestingly, adsorption of carbenicillin to the positively charged wall of the pore blocks the channel lumen only partially. These results demonstrate that not only the stericity (physical pore size and antibiotic size) but also the polarity of the drug critically affect its ability to bind near the pore constriction and to obstruct the channel lumen.

We also performed additional metadynamics simulations (1.5 ns) to explore the free energy surface for each antibiotic over a wide region within the upper part of the channel. As in the previous study (39), we calculated the free energy with respect to the molecule position along the z axis and the number of its hydrogen bonds with the channel. The initial conformations of the drugs corresponded to their center of mass along the z axis located at 7 Å, near the constriction zone ($z = 0$–4 Å) as in the standard simulations. The calculated free energy surfaces are reported in Fig. 6; they show that the barrier to escape from the binding sites is of the order

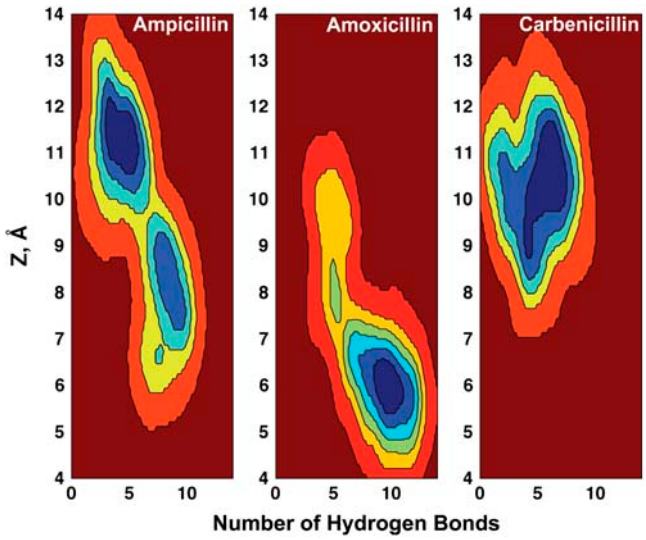


FIGURE 6 Contour plot of the free energy surfaces as obtained from metadynamics simulations with ampicillin (*left*), amoxicillin (*middle*), and carbenicillin (*right*). We used the number of hydrogen bonds and the position of the center of mass of the drug along the z axis as variables to characterize interactions with the channel (39). The difference between each successive color is 1 kcal/mol.

of 5 kcal/mol for each molecule. Specific binding regions with two pronounced sites for ampicillin and one for the other two drugs along the z axis can be observed. For ampicillin and amoxicillin we have found binding regions characterized by 5–10 hydrogen bonds at a position near the constriction zone between 5 and 9 Å. In contrast, carbenicillin finds interacting partners far from the constriction zone and with a lower number of hydrogen bonds. The new minimum is very similar to that shown in Fig. 5, with carbenicillin stuck to the positive wall but with reversed orientation, i.e., the COO^- close to the phenyl interacts with Arg-167 and Arg-168 and the COO^- on the penam ring binds to Arg-132. This reflects the increased affinity of ampicillin and amoxicillin for the charged amino acids near the constriction zone compared to carbenicillin that lacks the permanent dipole. Moreover, away from the constriction zone the number of hydrogen bonds decreases for both ampicillin and amoxicillin. Different interactions take place there, with only one terminal group of the drug molecules interacting with the channel wall. Since the standard MD simulations only sampled states in the vicinity of the initial position, these configurations could not be explored.

We have verified that the description of the system is relevant for different collective coordinates and initial conformations. To this end, we performed longer metadynamics simulations (6 ns) for each antibiotic and calculated the free energy as a function of three variables starting with the drugs placed away from the constriction zone ($z = 11$ Å). The first variable is the position of the drug, as above, and the second is its orientation (defined along the specific NH_3^+ group for ampicillin and amoxicillin or the COO^- group for carbenicillin and the common COO^- group) with respect to the channel axis, and the third is the central dihedral angle. With these parameters we found a minimum at $z = 6$–7 Å for ampicillin and amoxicillin but not for carbenicillin, which is stabilized at $z = 8$–10 Å from the constriction zone. The interaction networks for the three compounds are as described above. Moreover, changing the parameters and collective coordinates in the metadynamics described in Fig. 4 confirmed the mechanism of ampicillin translocation. Therefore, the results corroborate our previous observations on the structural prerequisites for a penicillin molecule to bind near the OmpF constriction zone, thus confirming the efficiency of the metadynamics methodology to provide accurate information on the properties of such complex systems.

Once ampicillin and amoxicillin have reached the energetically favorable region at $z = 6$–7 Å, the next step of the translocation process is to cross the constriction zone. We studied the free-energy barrier for amoxicillin overcoming the constriction zone by performing a metadynamics simulation of the net transport from the extracellular to the intracellular side of the channel. The free-energy surface explored by ampicillin (simulation time 3.0 ns) and amoxicillin (simulation time 2.2 ns) is shown in Fig. 7. The translocation mechanism is very similar for both drugs. However, the barrier for crossing the constriction zone for amoxicillin is ~3 kcal/mol lower than the one found for ampicillin. Thus our result suggests that amoxicillin crosses this zone of the channel faster than ampicillin.

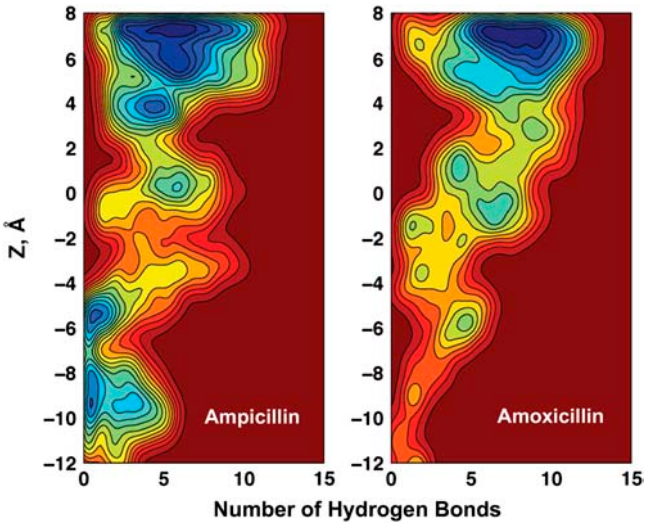


FIGURE 7 Contour plot of the free energy surfaces calculated for metadynamics simulations of ampicillin (*left*) and amoxicillin (*right*) translocations from the extracellular side to the intracellular side of the OmpF channel. The difference between each successive color is 1 kcal/mol.

DISCUSSION

MD simulations versus reconstitution experiments

Analysis of the drug-induced fluctuations in the ionic current through OmpF channels from *E. coli* reveals that the studied antibiotics interact with the pore center region along the following strength series: carbenicillin, azlocillin < piperacillin < amoxicillin < ampicillin (Figs. 1 and 2). Understanding which chemical groups of the antibiotic molecules are involved in the binding to the OmpF channel is important for predicting the ability of other drugs to translocate through the pore.

Recent simulation results (39) have revealed the main chemical groups of the zwitterionic ampicillin that are involved in interactions with the channel residues at the eyelet region. According to the findings of our study, ampicillin and amoxicillin have the strongest interaction. MD simulations performed for these two molecules demonstrate that the zwitterionic form of the β-lactam antibiotics is of principal importance for the strong binding to the protein interior (Figs. 5 and 6). The dianionic carbenicillin cannot be accommodated in the middle of the channel because simultaneous salt bridging with the opposite walls of the pore is not feasible (Fig. 5). Therefore, it is not surprising that nondipolar antibiotics of a size close to ampicillin's, such as carbenicillin,

display only poor binding to the OmpF constriction (Fig. 1 *B*). In addition, the aromatic group of ampicillin and amoxicillin also gives favorable energetic contribution during the translocation by interacting with hydrophobic environments. The hydroxyl group of amoxicillin does not alter interactions of the drug at the channel constriction since the plane of the aromatic is parallel to the wall (Fig. 5).

For ampicillin we find an extended binding site in the upper region, the phenyl group can stay either between Tyr-22 and Tyr-40 or between Tyr-22 and Tyr-32. Amoxicillin has a very stable binding site with the phenyl group close to Tyr-22 and Tyr-40 and OH interacting with the neighboring amino acids. Compared to these two antibiotics, dianionic carbenicillin cannot find a well-defined binding site near the constriction zone, so that its translocation is not facilitated by favorable interactions. Carbenicillin moves away from the constriction at the channel center to the positively charged regions interacting with both carboxylate groups. The strength of this binding is comparable to those obtained for ampicillin and amoxicillin. These results suggest that not only does the time-resolved channel occlusion imply strong binding, but the affinity site has to be as close as possible to the constriction zone. The latter condition is fulfilled for zwitterionic ampicillin and amoxicillin, but not for dianionic compounds like carbenicillin.

Our metadynamics simulations show that the translocation mode is the same for ampicillin and amoxicillin antibiotics (Fig. 7). The free-energy landscapes are also comparable, though the activation barrier for crossing the constriction zone by amoxicillin is found to be lower. These findings are in qualitative agreement with experiment. By extrapolating the values of ampicillin and amoxicillin residence time to zero voltage (Fig. 3 *B*), only a small difference in the depth of potential wells is expected.

Strength of interaction at the channel constriction correlates with permeation rate

In the absence of significant particle-channel interactions and at zero applied voltage, the particle average translocation time (53) is close to the characteristic diffusion relaxation time, $L^2/12D$ (52). Calculating this ''free diffusion estimate'' with $L \approx 5 \times 10^{-9}$ m as the channel length and $D \approx 3 \times 10^{-10}$ m^2/s as the diffusion coefficient of a nanometer-sized particle, we find that the characteristic time is in the range of nanoseconds. However, because of the attractive interactions between the particle and the channel this time is often orders of magnitude larger (50). This is exactly what we find in our study: for both ampicillin and amoxicillin the characteristic time is ~5 orders of magnitude larger than the free diffusion estimate given above, suggesting a strong interaction of these drugs with OmpF.

Despite the increasing frequency of amino penicillin resistant bacterial strains, ampicillin is still ''a drug of choice'' for treatment of *E. coli* infections in many cases (54,55). This antibiotic has been shown to bind to the OmpF channel during the translocation process rather than freely diffusing through it (16). To reveal a possible connection between the permeation rate, the 3D structure of the drugs, and the strength of OmpF-antibiotic binding, we have screened several other β-lactam antibiotics that have different in vitro diffusion rates. We find that

1. Carbenicillin, azlocillin, and piperacillin have a negligible binding to the narrowest part of the OmpF pore since they induce no significant modification in the flow of ions through the channel. These compounds have been shown to possess a very low rate of diffusion with the OmpF-containing liposomes (see Table 1, column 6) (14).
2. Ampicillin and amoxicillin have a pronounced affinity to OmpF since they induce time-resolved channel blockages. Ampicillin, along with several other zwitterionic compounds has been shown to induce higher swelling rates compared with carbenicillin and piperacillin in the OmpF-containing liposomes (14).

Therefore, we conclude that for the investigated β-lactam antibiotics, stronger interactions correlate with the enhanced diffusion through the OmpF channel.

It is well known that bacteria have evolved to create sophisticated substrate-specific channels, such as the maltodextrin-specific maltoporin, to bind corresponding substrates to facilitate their transport into the cell. Translocation through membrane channels via internal binding is more efficient than free diffusion at low substrate concentration. Analytical considerations of particle translocation through a channel (53,56) demonstrate that the presence of a potential well makes the channel more efficient. An optimal depth of the well depends on particle concentration (51). By assuming an extracellular concentration of antibiotics of several micromolar (14), the presence of an internal affinity zone with half-saturation constant similar to those found for the ampicillin-OmpF binding reaction (16) improves the rate of penetration through the outer membrane. In fact, the antibiotic concentration used in our study (5.7 mM) is ~3 orders of magnitude higher than those used in pharmaceutics. According to Nathwani and Wood (54), the peak serum concentration for ampicillin is 2–6 mg/L (~10 μM) two hours after a single 500 mg dose. These concentrations are far below the equilibrium dissociation constant between OmpF and the studied penicillins. Therefore, the conclusions we are drawing about the molecular mechanism of antibiotics transport can unambiguously be extrapolated from our 5.7 mM to much lower, clinically relevant concentrations.

Strong interactions at the channel constriction compensate for the entropy loss and the desolvation energy associated with the passage of compounds whose size is close to the size of the narrowest cross section of the pore. Zwitterionic ampicillin and amoxicillin clearly adopt this molecular mechanism to pass through OmpF. Although the low permeation

rate of azlocillin and piperacillin can be attributed to their bulky lateral chain, the poor conduction efficiency for carbenicillin probably originates from an unfavorable charge distribution at the channel eyelet, even if electrostatic interactions still take place in the extracellular vestibule. Therefore, one can rationalize the transit efficiency in terms of strength of the interaction between β-lactam antibiotics and the OmpF channels. We conclude that strong attractive interactions in the middle of the channel facilitate drug translocation resulting in potentially higher efficiency.

Carbenicillin, azlocillin, and piperacillin may penetrate into *E. coli* through channels of other porins, namely OmpC and PhoE (14,15). Even if the relevance of PhoE to chemotherapy has been questioned (14), this anion-selective porin may provide a favorable path for monoanionic azlocillin and piperacillin, and, to a larger extent, for dianionic carbenicillin (15). Deeper understanding of the molecular processes by which antibacterial agents cross the outer membrane will also require the study of porins from bacterial strains identified as pathogens, such as *Enterobacter cloacae* (57), *Enterobacter aerogenes* (10), and *Klebsiella pneumoniae* (58).

A number of important parameters other than just the rate of penetration into the periplasmic space modulate the antibacterial activity of penicillin drugs. In particular, the periplasmic concentration of β-lactams depends on the rate of their degradation by β-lactamases, which constitutes a resistance mechanism. The most permeable drugs may be the least stable toward β-lactamases, and thus inefficient. Moreover, now it is established that the intrinsic drug resistance of Gram-negative bacteria is a result of the cooperation between the outer membrane barrier and the expression of broad-specificity multidrug efflux pumps (4). Interestingly, one can observe a strong analogy in the structure involved in ampicillin stabilization in the active site of zinc β-lactamase (59) and in ampicillin binding to the OmpF channel. Clear structural similarities also exist between the binding site of the TEM-1 β-lactamase (60) and the constriction zone of the OmpF porin. One would expect that the most permeable penicillins are the most recognizable by these enzymes. Note that ampicillin and amoxicillin are used in combination with powerful β-lactamase inhibitors, sulbactam and clavulanic acid, respectively.

In conclusion, the diversity of outer membrane channels and periplasmic modulators of β-lactam concentration with the bacterial strains makes it difficult to establish direct correlations between the diffusion rate of the investigated drugs through OmpF and their bactericidal activity. Nevertheless, our study addresses one of the important aspects of antibiotic efficacy, which can be understood in terms of basic molecular interactions of permeating solutes with the membrane channels.

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REFERENCES

1. Kunin, C. M. 1993. Resistance to antimicrobial drugs—a worldwide calamity. *Ann. Intern. Med.* 118:557–561.
2. Levy, S. B. 1997. Antibiotic resistance: an ecological imbalance. *Ciba Found. Symp.* 207:1–9.
3. Normark, B. H., and S. Normark. 2002. Evolution and spread of antibiotic resistance. *J. Intern. Med.* 252:91–106.
4. Li, X.-Z., and H. Nikaido. 2004. Efflux-mediated drug resistance in bacteria. *Drugs.* 64:159–204.
5. Lakaye, B., A. Dubus, S. Lepage, S. Groslambert, and J.-M. Frere. 1999. When drug inactivation renders the target irrelevant to antibiotic resistance: a case story with β-lactams. *Mol. Microbiol.* 31: 89–101.
6. Tipper, D. J., and J. L. Strominger. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proc. Natl. Acad. Sci. USA.* 54:1133–1141.
7. Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* 67:593–656.
8. Delcour, A. H. 2003. Solute uptake through general porins. *Front. Biosci.* 8:d1055–d1071.
9. Nikaido, H. 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemother.* 33:1831–1836.
10. Chevalier, J., J.-M. Pages, and M. Mallea. 1999. In vivo modification of porin activity conferring antibiotic resistance to *Enterobacter aerogenes*. *Biochem. Biophys. Res. Commun.* 266:248–251.
11. Simonet, V., M. Mallea, and J.-M. Pages. 2000. Substitutions in the eyelet region disrupt cefepime diffusion through the Escherichia coli OmpF channel. *Antimicrob. Agents Chemother.* 44:311–315.
12. Nikaido, H., and S. Normark. 1987. Sensitivity of E.coli to various β-lactams is determined by the interplay of outer membrane permeability and degradation by the periplasmic β-lactamases: a quantitative predictive treatment. *Mol. Microbiol.* 1:29–36.
13. Nikaido, H., and E. Y. Rosenberg. 1983. Porin channels in *E. coli*: studies with liposomes reconstituted from purified proteins. *J. Bacteriol.* 153:241–252.
14. Yoshimura, F., and H. Nikaido. 1985. Diffusion of β-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* 27:84–92.
15. Nikaido, H., E. Y. Rosenberg, and J. Foulds. 1983. Porin channels in *E. coli*: studies with β-lactams in intact cells. *J. Bacteriol.* 153:232–240.
16. Nestorovich, E. M., C. Danelon, M. Winterhalter, and S. M. Bezrukov. 2002. Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. *Proc. Natl. Acad. Sci. USA.* 99:9789–9794.
17. Bezrukov, S. M., L. Kullman, and M. Winterhalter. 2000. Probing sugar translocation through maltoporin at the single channel level. *FEBS Lett.* 476:224–228.
18. Kullman, L., M. Winterhalter, and S. M. Bezrukov. 2002. Transport of maltodextrins through maltoporin: a single-channel study. *Biophys. J.* 82:803–812.
19. Danelon, C., T. Brando, and M. Winterhalter. 2003. Probing the orientation of reconstituted maltoporin channels at the single-protein level. *J. Biol. Chem.* 278:35542–35551.
20. Hou, J. P., and J. W. Poole. 1969. The amino acid nature of ampicillin and related penicillins. *J. Pharm. Sci.* 58:1510–1515.
21. Rolinson, G. N., and S. Stevens. 1961. Microbiological studies on a new broad-spectrum penicillin, ''penbritin''. *BMJ.* 2:191–196.

22. Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch. 1992. Crystal structures explain functional properties of two *E. coli* porins. *Nature*. 358:727–733.

23. Karshikoff, A., V. Spassov, S. W. Cowan, R. Ladenstein, and T. Schirmer. 1994. Electrostatic properties of two porin channels from *Escherichia coli*. *J. Mol. Biol.* 240:372–384.

24. Bransburg-Zabary, S., E. Nachliel, and M. Gutman. 2002. A fast in silico simulation of ion flux through the large-pore channel proteins. *Biophys. J.* 83:3001–3011.

25. Varma, S., and E. Jakobsson. 2004. Ionization states of residues in OmpF and mutants: effects of dielectric constant and interactions between residues. *Biophys. J.* 86:690–704.

26. Alcaraz, A., E. M. Nestorovich, M. Aguilella-Arzo, V. M. Aguilella, and S. M. Bezrukov. 2004. Salting out the ionic selectivity of a wide channel: the asymmetry of OmpF. *Biophys. J.* 87:943–957.

27. Watanabe, M., J. Rosenbusch, T. Schirmer, and M. Karplus. 1997. Computer simulations of the OmpF porin from the outer membrane of *Escherichia coli*. *Biophys. J.* 72:2094–2102.

28. Tieleman, D. P., and H. J. C. Berendsen. 1998. A molecular dynamics study of the pores formed by *Escherichia coli* OmpF porin in a fully hydrated palmitoyloleoylphosphatidylcholine bilayer. *Biophys. J.* 74: 2786–2801.

29. Robertson, K. M., and D. P. Tieleman. 2002. Molecular basis of voltage gating of OmpF porin. *Biochem. Cell Biol.* 80:517–523.

30. Suenaga, A., Y. Komeiji, M. Uebayasi, T. Meguro, M. Saito, and I. Yamato. 1998. Computational observation of an ion permeation through a channel protein. *Biosci. Rep.* 18:39–48.

31. Phale, P. S., A. Philippsen, C. Widmer, V. P. Phale, J. P. Rosenbusch, and T. Schirmer. 2001. Role of charged residues at the OmpF porin channel constriction probed by mutagenesis and simulation. *Biochemistry.* 40:6319–6325.

32. Im, W., and B. Roux. 2002. Ions and counterions in a biological channel: a molecular dynamics simulation of OmpF porin from *Escherichia coli* in an explicit membrane with 1 M KCl aqueous salt solution. *J. Mol. Biol.* 319:1177–1197.

33. Im, W., and B. Roux. 2002. Ion permeation and selectivity of OmpF porin: a theoretical study based on molecular dynamics, Brownian dynamics, and continuum electrodiffusion theory. *J. Mol. Biol.* 322: 851–869.

34. Danelon, C., A. Suenaga, M. Winterhalter, and I. Yamato. 2003. Molecular origin of cation selectivity in OmpF porin: single channel conductances versus free energy calculation. *Biophys. Chem.* 104:591–603.

35. Isralewitz, B., M. Gao, and K. Schulten. 2001. Steered molecular dynamics and mechanical functions of proteins. *Curr. Opin. Struct. Biol.* 11:224–230.

36. Laio, A., and M. Parrinello. 2002. Escaping free-energy minima. *Proc. Natl. Acad. Sci. USA.* 99:12562–12566.

37. Jensen, M. O., S. Park, E. Tajkhorshid, and K. Schulten. 2002. Energetics of glycerol conduction through aquaglyceroporin GlpF. *Proc. Natl. Acad. Sci. USA.* 99:6731–6736.

38. Robertson, K. M., and D. P. Tieleman. 2002. Orientation and interactions of dipolar molecules during transport through OmpF porin. *FEBS Lett.* 528:53–57.

39. Ceccarelli, M., C. Danelon, A. Laio, and M. Parrinello. 2004. Microscopic mechanism of antibiotics translocation through a porin. *Biophys. J.* 87:58–64.

40. Nestorovich, E. M., T. K. Rostovtseva, and S. M. Bezrukov. 2003. Residue ionization and ion transport through OmpF channels. *Biophys. J.* 85:3718–3729.

41. Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA.* 69:3561–3566.

42. Bezrukov, S. M., and I. Vodyanoy. 1993. Probing alamethicin channels with water-soluble polymers. Effect on conductance of channel states. *Biophys. J.* 64:16–25.

43. Rostovtseva, T. K., E. M. Nestorovich, and S. M. Bezrukov. 2002. Partitioning of differently sized poly(ethylene glycol)s into OmpF porin. *Biophys. J.* 82:160–169.

44. Ceccarelli, M., and M. Marchi. 2003. Simulation and modeling of the *Rhodobacter sphaeroides* bacterial reaction center: structure and interactions. *J. Phys. Chem. B.* 107:1423–1431.

45. Procacci, P., T. A. Darden, E. Paci, and M. Marchi. 1997. ORAC: a molecular dynamics program to simulate complex molecular systems with realistic electrostatic interactions. *J. Comput. Chem.* 18:1848–1862.

46. Petrauskas, A. A., and V. K. Svedas. 1991. Hydrophobicity of β-lactam antibiotics. Explanation and prediction of their behaviour in various partitioning solvent systems and reversed-phase chromatography. *J. Chromatogr.* 585:3–34.

47. DeFelice, L. J. 1981. Introduction to Membrane Noise. Plenum Press, New York.

48. Nekolla, S., C. Andersen, and R. Benz. 1994. Noise analysis of ion current through the open and the sugar-induced closed state of the LamB channel of *Escherichia coli* outer membrane: evaluation of the sugar binding kinetics to the channel interior. *Biophys. J.* 66:1388–1397.

49. Bezrukov, S. M., I. Vodyanoy, and V. A. Parsegian. 1994. Counting polymers moving through a single ion channel. *Nature.* 370:279–281.

50. Bezrukov, S. M. 2000. Ion channels as molecular Coulter counters to probe metabolite transport. *J. Membr. Biol.* 174:1–13.

51. Berezhkovskii, A. M., and S. M. Bezrukov. 2005. Optimizing transport of metabolites through large channels: molecular sieves with and without binding. *Biophys. J.* 88:L17–L19.

52. Feher, G., and M. Weissman. 1973. Fluctuation spectroscopy: determination of chemical reaction kinetics from the frequency spectrum of fluctuations. *Proc. Natl. Acad. Sci. USA.* 70:870–875.

53. Berezhkovskii, A. M., M. A. Pustovoit, and S. M. Bezrukov. 2003. Channel-facilitated membrane transport: Average lifetimes in the channel. *J. Chem. Phys.* 119:3943–3951.

54. Nathwani, D., and M. J. Wood. 1993. Penicillins. A current review of their clinical pharmacology and therapeutic use. *Drugs.* 45:866–894.

55. Fairbanks, D. N. F. 2001. Pocket Guide to Antimicrobial Therapy in Otolaryngology—Head and Neck Surgery. American Academy of Otolaryngology, Alexandria, VA.

56. Berezhkovskii, A. M., M. A. Pustovoit, and S. M. Bezrukov. 2002. Channel-facilitated membrane transport: transit probability and interaction with the channel. *J. Chem. Phys.* 116:9952–9956.

57. Chevalier, J., M. Mallea, and J.-M. Pages. 2000. Comparative aspects of the diffusion of norfloxacin, cefepime and spermine through the F porin channel of *Enterobacter cloacae*. *Biochem. J.* 348:223–227.

58. Chevalier, J., J.-M. Pages, A. Eyraud, and M. Mallea. 2000. Membrane permeability modifications are involved in antibiotic resistance in *Klebsiella pneumoniae*. *Biochem. Biophys. Res. Commun.* 274:496–499.

59. Krauss, M., N. Gresh, and J. Antony. 2003. Binding and hydrolysis of ampicillin in the active site of a zinc lactamase. *J. Phys. Chem. B.* 107:1215–1229.

60. Ness, S., R. Martin, A. M. Kindler, M. Paetzel, M. Gold, S. E. Jensen, J. B. Jones, and N. C. J. Strynadka. 2000. Structure-based design guides the improved efficacy of deacylation transition state analogue inhibitors of TEM-1 β-lactamase. *Biochemistry.* 39:5312–5321.

61. Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: visual molecular dynamics. *J. Mol. Graph.* 14:33–38.